Inhibition of nitric oxide synthase does not prevent ocular dominance plasticity in kitten visual cortex

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1. The neural messenger molecule nitric oxide (NO) has been shown to be involved in several forms of plasticity including hippocampal long-term potentiation. We examined the effects of chronic intracortical infusion of inhibitors of NO synthase (NOS) activity on the plasticity of visual cortical responses following monocular lid suture during the critical period.

2. Single unit recordings (618 cells) made in both the NOS inhibitor-treated (30 mM N^G-methyl-L-arginine (L-NMMA), or 22 or 2 mM nitro-L-arginine (L-NOArg)) and saline-treated control hemispheres of barbiturate-anaesthetized, critical-period kittens ($n = 8$) revealed a profound shift in favour of the non-deprived eye. Shifts were of similar magnitude in hemispheres in which NOS was inhibited and in saline control hemispheres.

3. Subsequent analysis of NOS activity in the same cortical tissue in which recordings had been made showed a pronounced decrease in NOS activity in inhibitor-treated hemispheres. In the region in which all the single unit recordings were made (< 3 mm from the infusion cannula), 22 mM L-NOArg resulted in a reduction of NOS activity to 5·55 ± 5·33% of control hemisphere NOS activity levels. L-NOArg (2 mM) and L-NMMA (30 mM) also produced clear, but smaller, inhibition of NOS activity.

4. These findings demonstrate that NOS activity is not essential for ocular dominance plasticity in visual cortex.

During a period in the early postnatal life of the cat, the visual cortex exhibits great plasticity in response to manipulations of visual experience. Brief occlusion of vision in one eye during this period causes the initially binocular visual cortex to respond almost exclusively to stimulation of the unoccluded eye (Olson & Freeman, 1975; Shatz & Stryker, 1978; Wiesel, 1982; Hensch, Crair, Ruthazer, Fagiolini, Gillespie & Stryker, 1995). Anatomically, cortical territory receiving input from the deprived eye shrinks and individual deprived geniculocortical axon arbors show rapid decreases in arbor complexity (Shatz & Stryker, 1978; Wiesel, 1982; Antonini & Stryker, 1993*b*). Simple atrophy of inactive inputs cannot account for ocular dominance plasticity because even much longer periods of binocular deprivation or binocular retinal activity blockade do not cause an appreciable decrease in cortical responsiveness or thalamocortical arbor coverage (Wiesel, 1982; Stryker & Harris, 1986; Antonini & Stryker, 1993*a*).

Activity of the postsynaptic cortical cell is both permissive and instructive for ocular dominance plasticity. Blockade of cortical activity reversibly prevents ocular dominance plasticity (Reiter, Waitzman & Stryker, 1986). Chronic application of the *N*-methyl-D-aspartate (NMDA) receptor antagonist amino-phosphonovaleric acid (APV) greatly attenuates the ocular dominance shift in response to monocular deprivation (Bear, Kleinschmidt, Gu & Singer, 1990), although the chronic application of APV *in vivo* has additional effects on responsiveness that complicate interpretation (Miller, Chapman & Stryker, 1989). In addition, selectively inhibiting postsynaptic cortical cells

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by chronically infusing the $GABA_A$ receptor agonist muscimol into the cortex effectively reverses the direction of both the electrophysiological and anatomical expressions of ocular dominance plasticity (Hata & Stryker, 1994).

A modified Hebbian model based on the competitive strengthening or weakening of inputs that are, respectively, correlated or anti-correlated in their firing pattern with the postsynaptic neuron is consistent with all these observations (Miller, Keller & Stryker, 1989). With this model, the rapid changes in the structure of presynaptic axon arbors would occur only when the postsynaptic target cell detected changes in their degree of mutual correlation. This requires the existence of a retrograde signal by which the postsynaptic cell informs presynaptic axons whether to grow or shrink.

The freely diffusible gaseous neurotransmitter nitric oxide (NO) has been proposed as an intercellular messenger for some cellular models of associative synaptic plasticity such as hippocampal long-term potentiation (LTP) and cerebellar long-term depression (LTD) (reviewed in Schuman & Madison, 1994; Garthwaite & Boulton, 1995). NOS inhibitors or haemoglobin, which absorbs ambient NO, can greatly decrease the probability of generating certain forms of LTP in the CA1 field of the hippocampus (O'Dell, Hawkins, Kandel & Arancio, 1991; but see Cummings, Nicola & Malenka, 1994; reviewed in Schuman & Madison, 1994). The paired application of exogenous sources of NO and electrical stimulation can augment CA1 responses and has been shown to increase the frequency of quantal release of cultured hippocampal neurons (O'Dell *et al.* 1991; Zhuo, Small, Kandel & Hawkins, 1993).

The ready diffusibility, membrane permeability and short half-life of NO, as well as the regulation of the neuronal form of NO synthase (NOS) by intracellular calcium (for reviews see Moncada, Palmer & Higgs, 1991; Dawson & Snyder, 1994) make NO a suitable candidate for the retrograde signal predicted by the Hebbian model. NOS is present in a subset of cells throughout the neocortex (Bredt, Glatt, Hwang, Foruhi, Dawson & Snyder, 1991) and has been demonstrated to underlie enhanced glutamate release in cortical synaptosomes following NMDA receptor stimulation (Montague, Gancayco, Winn, Marchase & Friedlander, 1994, but see Kirkwood & Bear, 1994, their Fig. 5b). Moreover, the distribution of NOS in visual cortex can be altered by manipulation of visual inputs (Aoki, Fenstemaker, Lubin & Go, 1993). The properties of NO and the results cited above prompted us to consider NO as a possible retrograde signal in visual cortical plasticity.

In the present study, we have taken advantage of the availability of potent, selective inhibitors of NOS together with sensitive assays for NOS activity to determine whether NO is the intercellular messenger responsible for ocular dominance plasticity.

METHODS

Minipump implantation and monocular deprivation

Normal kittens ranging in age from 24 to 35 days (see Table 1) were anaesthetized using halothane (0·5–2%) and nitrous oxide. Under sterile surgical conditions, two subcutaneous osmotic minipumps (model 2001 or 2002, Alza Corp., Palo Alto, CA, USA) attached to intracerebral 30 gauge cannulae were stereotaxically implanted to deliver drug and control solutions bilaterally into primary visual cortex 2 mm below the cortical surface and 2 mm lateral to the mid-line at Horsley-Clarke AP 0.0. As drug and saline control solutions were prepared under identical conditions and encoded prior to surgery, it was possible in six animals (cats 3–8) to perform the surgery and all following procedures without knowing which hemisphere received inhibitor. In two animals (cats 1 and 2), in which only one cannula was implanted, the experiment was not performed blind. Drug infusion solutions were prepared by warming sterile saline to just below 37 °C and adjusting pH as close to 7·4 as possible while stirring in one of the following agents to achieve the following concentrations: 30 mM N^G-methyl-L-arginine (L-NMMA), or 2 or 22 mM *N*-nitro-L-arginine (L-NOArg) (both from Sigma).

After 2 days of infusion, allowing the affected area of cortex to attain a stable size, the right eye of each animal was sutured shut under aseptic conditions using the same anaesthetic protocol as above. Animals were then returned to their littermates to experience monocular vision for a period of 4–9 days.

Electrophysiology

Animals were premedicated with atropine (0·1 mg kg^{-1}, s.c.) to control secretions and were prepared for physiological recording by inserting an intravenous cannula (50 gauge polyethylene tubing) into the femoral vein under halothane anaesthesia (0·5–2%). Anaesthesia was subsequently maintained by intravenous administration of pentobarbitone sodium, delivered to effect a tonic state of muscle relaxation in a series of boluses of about 4 mg kg^{-1} to a total surgical dose of 20–40 mg kg^{-1} in the first 1–2 h. A tracheal cannula was inserted, following which animals breathed a 2:1 $N_2O:O_2$ mixture. Animals were then placed in a stereotaxic head holder and on a feedback-controlled heating pad to maintain core temperature at 38 °C. The sutured eyelid was opened and the pupils of both eyes were dilated with a single application of atropine sulphate ophthalmic solution (1%). The nictitating membranes were retracted with a single application of phenylephrine hydrochloride ophthalmic solution (10%). A cranial opening exposing the visual cortex up to about 8 mm anterior to the cannulae in both hemispheres was drilled. Just prior to dura retraction and for the remainder of the experiment, animals were paralysed by intravenous infusion of gallamine triethiodide (10 mg kg^{-1} h^{-1}) in 5% dextrose Ringer solution (1–2 ml kg^{-1} h^{-1}) and artificially ventilated with a 2:1 $N_2O:O_2$ mixture at a rate and volume appropriate to maintain end-tidal expired CO_2 at 4·0 ± 0·5%. Contact lenses were selected to protect and focus both eyes on a tangent screen and the optic discs were refracted and plotted to insure that both eyes could be stimulated on the screen.

An adequate level of anaesthesia was maintained by giving additional barbiturate as determined by continuous monitoring of slow waves and spindles in posterior temporal supradural EEG recordings (bandpass filtered to 0·1–30 Hz) on a 10 s continuously scrolling display. End-tidal CO_2 levels and EKG (heart rate within the range of 180–260 beats min^{-1}) were also continuously monitored for stability as a fail-safe indicator that the animal was

Table 1. Experimental conditions and corresponding ocular dominance shifts

Animal	Infusion drug	Age (days)			Shift index (no. of cells)	
		Infusion onset	MD	Physiology	Inhibitor	Control
Cat 1	30 mM L-NMMA (0·5 μl h^{-1})	28	30	39	0·98 (30)	n.a.
Cat 2	30 mM L-NMMA (0·5 μl h^{-1})	28	30	36	0·99 (33)	n.a.
Cat 3	22 mM L-NOArg (1 μl h^{-1})	33	35	40	0·88 (37)	0·93 (54)
Cat 4	22 mM L-NOArg (1 μl h^{-1})	35	37	42	0·99 (47)	0·82 (45)
Cat 5	2 mM L-NOArg (1 μl h^{-1})	31	33	38	0·93 (61)	0·95 (37)
Cat 6	30 mM L-NMMA (1 μl h^{-1})	24	26	30	0·84 (46)	0·86 (46)
Cat 7	30 mM L-NMMA (1 μl h^{-1})	24	26	31	0·82 (44)	0·95 (46)
Cat 8	22 mM L-NOArg (1 μl h^{-1})	26	28	33	0·91 (46)	0·89 (46)

MD, age at monocular eyelid suture. Shift index, see Methods for definition.

at an appropriate anaesthetic level. Adherence to the above criteria rendered the animals completely areflexive to a firm pinch of the toe and to corneal touch prior to administration of paralytic drugs.

Single unit recordings were then made by advancing a tungsten microelectrode (1–3 MΩ impedance) down the medial bank of the lateral gyrus in 100 μm increments with a stepping-motor microdrive. The receptive field centre, orientation preference, ocular dominance, responsiveness and habituation of all units were determined by stimulating with a hand-held slit lamp. At least thirty units were characterized in each hemisphere. These protocols, including all surgery and experimental procedures, were approved by the University of California at San Francisco Committee on Animal Research.

For comparative analysis of the ocular dominance distribution of each hemisphere, the contralateral bias index (CBI) of Reiter *et al.* (1986) was adopted:

$$\mathrm{CBI} = 100 \times [(\mathbf{1–7}) + ((2/3)(\mathbf{2–6})) + ((1/3)(\mathbf{3–5}) + n]/2n,$$

where emboldened numbers equal the number of units in each ocular dominance group and n equals the total number of visually responsive units. This index has a value of 100 for a collection of neurons that responds exclusively to the contralateral eye, 0 for exclusively ipsilateral responses, and intermediate values for binocular responses. The 'shift index' used in this paper is defined as the CBI/100 for the hemisphere contralateral to the open eye and 1·00 − (CBI/100) for the hemisphere ipsilateral to the open eye. This permits a characterization of each hemisphere's ocular dominance distribution relative to the open eye. Although the two hemispheres are not perfectly equally driven by both eyes in normal animals, it is acceptable to compare the two hemispheres in this manner because (1) the distribution in normal kittens (CBI ≈ 55) is very close to symmetrical (CBI = 50), and (2) our blind procedure results in the experimental hemisphere and the deprived eye assorting independently.

Tissue collection for NOS assay

Immediately after finishing recording, animals were given a lethal dose of pentobarbitone sodium as monitored by EKG and were perfused transcardially with about 600 ml of ice-cold oxygenated phosphate-buffered saline. The craniotomy for recording was then expanded and the cannulae were removed. A 10 mm-long segment of the lateral gyri of both hemispheres was excised by making a lateral incision at the lateral sulcus, a ventral incision at the splenial sulcus, a posterior incision at the cannula insertion point and an anterior incision 10 mm anterior to the cannula insertion point incision. The 10 mm segments from both hemispheres were then placed in individual slicing moulds with a series of 1 mm-spaced slits for insertion of razor blades and rapidly embedded in 4% agarose in saline. Slices 1 mm thick were then taken in a caudal-to-rostral sequence, alternating between hemispheres to assure identical treatment of experimental and control hemispheres, and frozen on dry ice for storage at −80 °C.

Assay for NOS activity

NOS activity in a homogenate from each 1 mm slice was quantified as described in Bredt & Snyder (1989) by measuring the conversion of [^{3}H]arginine to [^{3}H]citrulline (New England Nuclear, Boston, MA, USA). In brief, following a 1 h spin at 100 000 *g*, 25 μl tissue supernatant was added to 100 μl buffer containing 50 mM Tris-HCl (pH 7·4), 1 mM NADPH, 1 mM EDTA, 1 mM EGTA, 2·25 mM $CaCl_2$ and 0·1 μCi [^{3}H]arginine, and incubated at 25 °C. After 15 min, 3 ml of buffer containing 20 mM Hepes (pH 5·5), 1 mM EDTA, and 1 mM EGTA was added to terminate the reaction. [^{3}H]citrulline was quantitated by liquid scintillation counting of the reaction solution eluate from 0·5 ml columns of Dowex AG50WX-8 (Sigma). Assays were carried out blind to prior treatment of the tissue. Each measurement was normalized to the total protein per sample. Although this assay does not measure absolute NOS activity, it does provide a quantitative measurement of relative enzymatic activity in different tissue samples.

RESULTS

Infusion of inhibitors of NOS fails to prevent ocular dominance plasticity

Single unit electrophysiological recordings from 618 neurons in eight kittens (referred to as cat 1 to cat 8) approximately 1 month old were made following about 1 week of continuous infusion at 0·5 or 1 μl h^{-1} of one of the following: 30 mM L-NMMA, 2 mM L-NOArg or 22 mM L-NOArg (see Table 1). The visual cortex in the other hemisphere of all kittens, except for cat 1 and cat 2, was infused in an identical manner except that sterile saline control solution was substituted for the drug. The labels on the pairs of vials of NOS inhibitor and control solutions were coded before surgery so that the investigators did not know which was the experimental and which was the control hemisphere until after the experiment was completed. Animals were monocularly deprived by suturing the right eyelid shut 2 days after the infusion cannulae were

implanted. We waited 2 days before manipulating visual input in order to allow the infusion drug to achieve a stable intracerebral concentration gradient. Electrophysiological recordings were made after a period ranging from 4 to 9 days of monocular lid suture, which reliably produces a strong shift of responsiveness in favour of the open eye (Olson & Freeman, 1975; Reiter *et al.* 1986; Hensch *et al.* 1995)

We were able to detect no consistent differences in the physical appearance of the cortex or cortical vasculature of the two hemispheres. Electrode penetrations were made down the medial bank of the visual cortex in the vicinity of the infusion cannula in both hemispheres. The median number of single units recorded in each penetration was fifteen, typically at 100 μm intervals. All penetrations were made within 2·5 mm along the anterioposterior axis from the cannula to insure that neurons recorded were located in the region affected by the drug infusion. Although the biochemical analysis precluded histological determination of the laminar distribution of cells in this study, we are confident that the population of cells in this study includes at least layers II to IV, as there was a well-defined range of depths in many penetrations in which thalamocortical afferent responses were easily recorded, as is typical of layer IV in area 17. Neurons were characterized for preferred orientation, quality of response, and relative ability of the two eyes to elicit a response (ranked according to the seven point scale of Hubel and Wiesel (see Wiesel, 1982)).

A strong ocular dominance shift towards the open eye was observed in both control and NOS-inhibited hemispheres of all animals studied. Figure 1 compares an ocular dominance histogram from normal kittens of this age with the grouped data from experimental and control

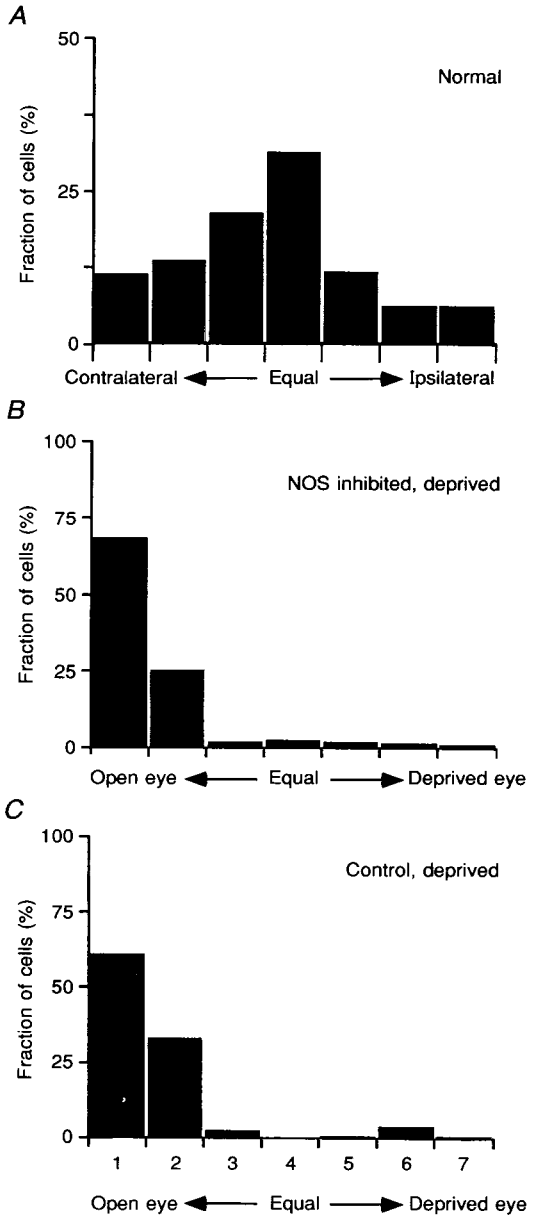

Figure 1. Ocular dominance histograms show a robust shift in monocularly deprived animals infused with NOS inhibitors or with saline

A shows the distribution of ocular dominance responses seen by Stryker & Harris (1986) in normal kittens of comparable age to those in this study (CBI = 0·57). Few cells (< 20%) are driven exclusively by one eye. The histograms in *B* and *C* show the distribution of ocular dominance of all the cells in this study from drug-treated (n = 344 cells, shift index = 0·92) and saline control (n = 274 cells, shift index = 0·90) hemispheres, respectively. The majority of cells recorded in monocularly deprived animals (> 65%) are driven exclusively by the open eye. This shift toward monocular responses is unaffected by inhibitors of NOS. As cannula implantation, eyelid suture and recording were performed blind with respect to minipump contents, Hubel & Wiesel's seven point contra-ipsi scale was modified for *B* and *C*, so that 1 represents cells driven purely by the non-deprived eye and 7 represents cells driven exclusively by the deprived eye.

hemispheres of deprived animals in this study. The overall ocular dominance distribution for a hemisphere can be quantified as a 'shift index', in which zero represents complete domination by the deprived eye and one represents responses exclusively driven by the open eye. The mean shift index for the confirmed NOS-inhibited visual cortices was 0·89 ± 0·05 (S.E.M., $n = 6$ animals), *versus* a shift index of 0·90 ± 0·02 (S.E.M., $n = 6$ animals) for control hemispheres. Ocular dominance distributions in control and experimental hemispheres of each animal, and of each group of animals pooled by experimental condition, were not found to be significantly different from each other (two-tailed Wilcoxon non-parametric test, $P > 0·05$), except in the case of cat 4 which showed a somewhat greater shift in the experimental hemisphere (0·99) than the control (0·82). Figure 2 illustrates ocular dominance histograms for each of the experimental conditions along with control data.

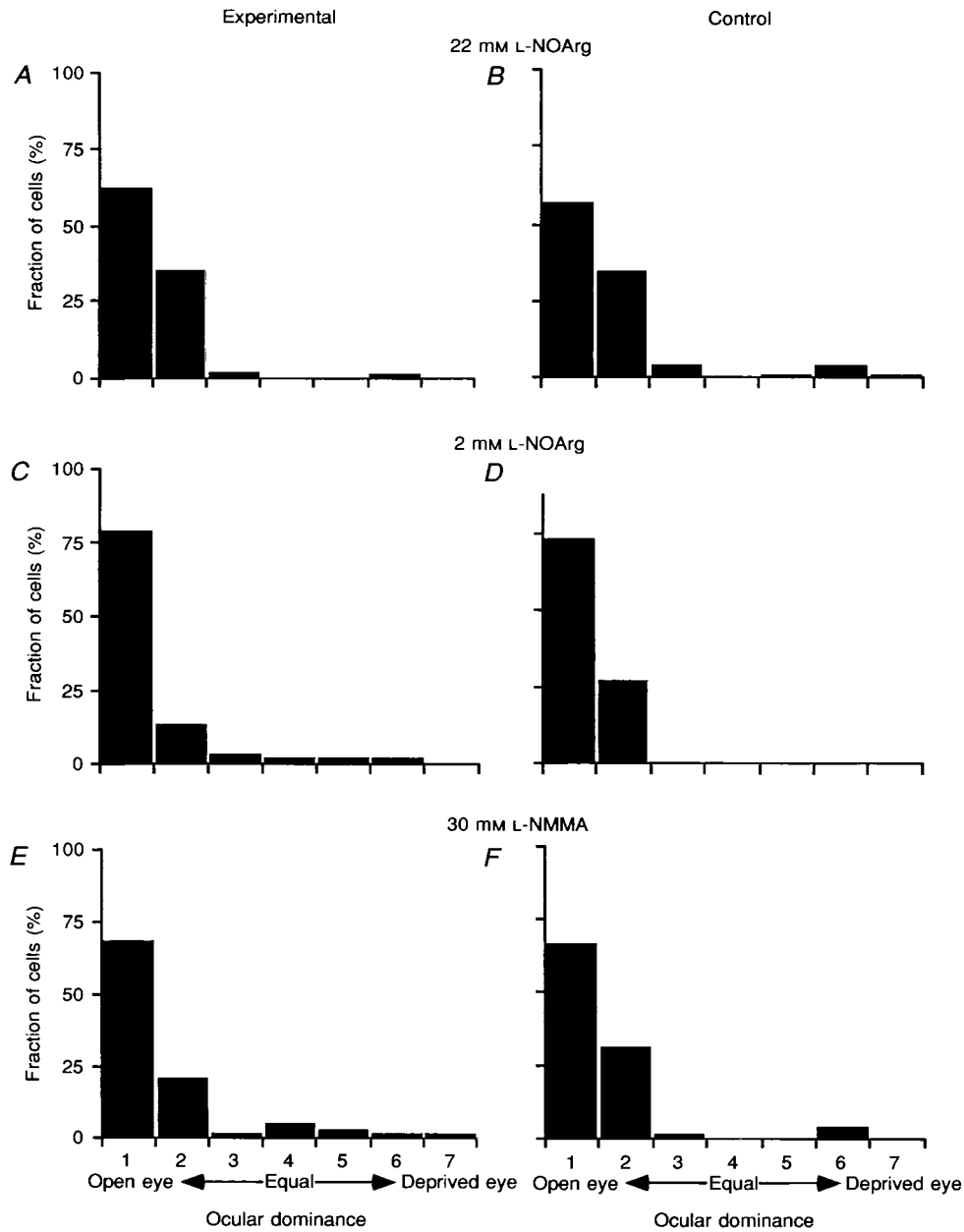

Figure 2. Ocular dominance histograms for each experimental condition

A, *C* and *E* show histograms of ocular dominance for single units recorded in regions treated with 22 mM L-NOArg ($n = 130$ cells in 3 animals), 2 mM L-NOArg ($n = 61$ cells in one animal), and 30 mM L-NMMA ($n = 153$ cells in 4 animals), respectively. *B* ($n = 145$), *D* ($n = 37$), and *F* ($n = 92$) are histograms from control hemispheres of the same animals in *A*, *C* and *E*, respectively. There is no significant difference between any of the groups and their controls.

There was also no significant difference in the responsiveness of cortical neurons to visual stimuli between control and experimental hemispheres, with 90% of NOS-inhibited neurons giving strong responses, 9% showing moderate responsiveness, and 1% responding poorly, compared with 85, 13 and 2%, respectively, in control penetrations. Neurons from both hemispheres displayed narrowly tuned orientation selectivity, with only 2·6% (9/344) of NOS-inhibited neurons and 1·8% (5/274) of control neurons completely lacking orientation tuning. While acute iontophoretic application of NOS inhibitor in the visual cortex has been reported to reversibly alter neuronal response properties in a subset of cells (Kara & Friedlander, 1995), it is not known whether these changes are due to local effects on vascular perfusion. We saw no evidence of altered responsiveness or orientation selectivity under conditions of profound, long-term blockade of NOS activity. Indeed, it was impossible on the basis of electrophysiological properties to deduce which hemisphere was infused with the NOS inhibitor.

Nitric oxide synthase activity in visual cortex is greatly reduced or eliminated by chronic infusion of inhibitor

In order to confirm that continuous infusion of L-NMMA or L-NOArg into visual cortex did produce a local inhibition of NOS activity, brain samples were taken for biochemical analysis at the conclusion of electrophysiology. Figure 3*A* illustrates the effects of 22 mM L-NOArg infusion on cortical NOS activity. In the 3 mm region closest to the cannula, from which all the ocular dominance data were derived, drug infusion decreased levels of NOS activity by an average of 94% below that in the comparable 3 mm segment of control cortex, and by 96% below the mean level for the entire control side. Moreover, in the case of cat 8, NOS activity was below detectable levels in the 2·5 mm region in which electrophysiological data had been collected. Similar results were obtained using 2 mM L-NOArg except that inhibition was slightly less pronounced (90% reduction for the first 3 mm nearest the cannula) and the region of greatest blockade was somewhat smaller (Fig. 3*B*).

There was considerable intra-animal variation in levels of NOS activity per milligram of total protein between different slices from the control hemisphere, which may perhaps reflect differences in the distribution of cerebral blood vessels. However, the fact that the sections nearest the L-NOArg cannulae showed very little variability in NOS activity (Fig. 3*A*) suggests that L-NOArg was actually above saturation inhibition concentration in the 2·5 mm-long region that had been electrophysiologically characterized. The actual concentration of L-NOArg would be expected to decrease steeply as a function of distance from the cannula, but NOS activity remained almost unchanged with regard to distance until more than 3 mm anterior to the cannula, where it first began to increase with distance.

L-NMMA was considerably less effective at locally inhibiting NOS activity in kitten visual cortex. NOS activity in the first 3 mm of the experimental hemispheres was 25 and

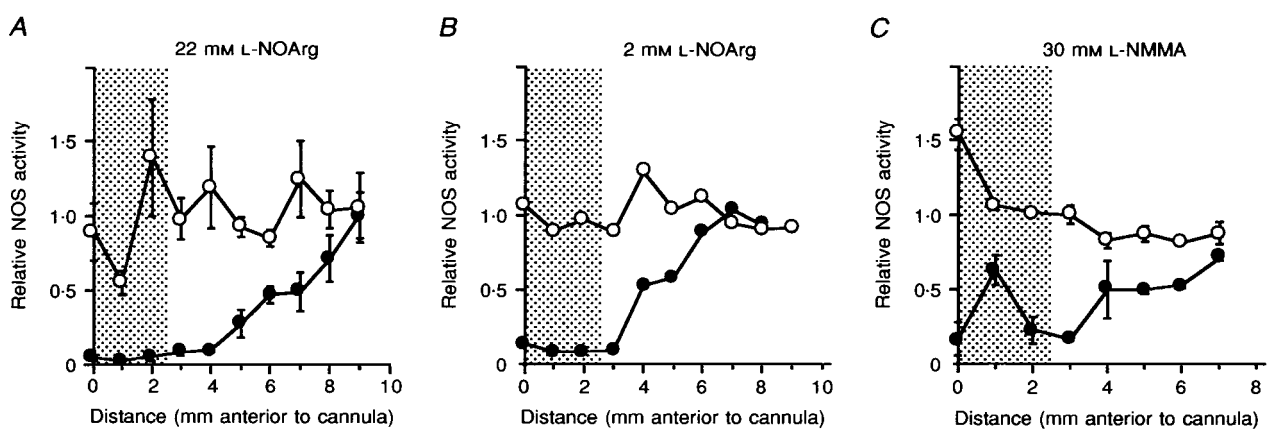

Figure 3. Normalized nitric oxide synthase activity as a function of distance from the infusion cannula

Chronic L-NOArg infusion at concentrations of 22 mM (*A*, $n = 3$ animals) and 2 mM (*B*, $n = 1$ animal) produces a region in which NOS activity is profoundly reduced. L-NMMA (30 mM; *C*, $n = 2$ animals) results in a significant, but smaller reduction. The points represent c.p.m. (μg protein)$^{-1}$ normalized within individual animals to the mean c.p.m. (μg protein)$^{-1}$ for all tissue sections taken from the control hemisphere (●, inhibitor-treated hemispheres; ○, control hemispheres; error bars are S.E.M.). This allows data from different animals to be pooled based on the assumption that the control hemisphere data represent baseline levels of NOS activity. Mean c.p.m. (μg protein)$^{-1}$ (× 100) ± S.D. (× 100) for each control hemisphere are: (*A*) cat 3, 606 ± 185; cat 4, 424 ± 183; cat 8, 306 ± 162; (*B*) cat 5, 220 ± 28; (*C*) cat 6, 810 ± 243; cat 7, 722 ± 140. The shaded area indicates the range of distances at which single unit recordings were made.

29% of the levels in the comparable control regions (Fig. 3C). This is consistent with reports from other groups that L-NOArg is a better inhibitor of the neuronal form of brain NOS than is L-NMMA (Dwyer, Bredt & Snyder, 1991).

DISCUSSION

These experiments reveal that normal, robust ocular dominance plasticity does occur in visual cortex in which NOS activity is pharmacologically blocked.

The demonstration of greatly reduced NOS activity in the same tissue from which electrophysiological data were collected ensures that the drugs used in this study accomplished their intended biochemical effect. Previous experiments using the same intracerebral infusion protocols with other drugs, having molecular weights similar to L-NOArg and L-NMMA, have clearly established that a 48 h period prior to eyelid suture is sufficient for steady-state concentrations of infusion drug to bathe the region of interest in the visual cortex (Reiter *et al.* 1986). The confirmation that NOS activity continued to be profoundly inhibited after 7 days of continuous infusion of inhibitor makes it highly unlikely that there might have been any window in time during the period of deprivation in which NOS was not inhibited.

While the biochemical assay in this study did not measure NO production directly, it did directly measure the catalytic activity of NOS for the production of the citrulline by-product of NO formation. It is conceivable that the inhibitors infused into cortex were somehow excluded from access to the NOS until the tissue was homogenized for the biochemical assay. This is improbable for two reasons: (1) experiments with NO-sensitive electrodes have directly demonstrated the ability of inhibitors to block NO production when applied extracellularly (Shibuki & Okada, 1991); (2) extracellular NOS inhibitor would have been washed out prior to freezing the tissue samples during the perfusion and sectioning.

The finding that normal ocular dominance plasticity, measured electrophysiologically, occurs in regions of visual cortex where NOS is completely inhibited, or nearly so, rules out any model that requires NO production as a necessary step for this form of cortical plasticity. Nonetheless it is impossible to exclude entirely any role for NO. An alternative explanation for our finding is that NO may be important for only one of several redundant or mutually facilitatory mechanisms that underlie ocular dominance plasticity. For example, homeostatic mechanisms have been shown to compensate for the absence of neuronal NOS in the regulation of cerebral blood flow in mice lacking neuronal NOS gene expression (Irikura *et al.* 1995).

If redundant mechanisms in ocular dominance plasticity were able to fully compensate for the absence of NOS activity, our assay would fail to detect an effect. On the other hand, if NO were involved in one of several facilitatory mechanisms, ocular dominance plasticity would be predicted to occur more slowly than normal without the contribution of the NO-dependent pathway. In an additional animal that was monocularly deprived for just 2 days, the minimum deprivation known to produce a consistent physiological shift, 22 mM L-NOArg did not impair ocular dominance plasticity ($n = 50$ cells, shift index = 0·88, data not shown) compared with control 2 day-deprived animals.

In addition to the studies that demonstrate a requirement for NO synthesis or release in hippocampal LTP, there have been numerous reports of NO-independent forms of LTP (Gribkoff & Lum-Ragan, 1992; Chetkovich, Klann & Sweatt, 1993; Haley, Malen & Chapman, 1993; Williams, Li, Naya, Errington, Muphy & Bliss, 1993; Cummings *et al.* 1994). The existence of NO-independent forms of LTP in the CA1 field of the hippocampus, however, does not rule out a contribution of NO in hippocampal plasticity. Systemic injection of NOS inhibitors has been reported to impair spatial learning in the Morris water maze (Chapman, Atkins, Allen, Haley & Steinmetz, 1992) and in the radial arm maze (Bohme *et al.* 1993), two tasks believed to rely on plasticity in the hippocampus. While the specificity of NOS inhibition on hippocampal learning has been contested (Bannerman, Chapman, Kelly, Butcher & Morris, 1994), the complete absence of any effect of NOS inhibition on ocular dominance plasticity suggests an even smaller role, if any, for NO in neocortical developmental plasticity. Indeed, no requirement for NO in visual cortical LTP has yet been reported.

LTP induced by electrical stimulation of neural pathways is an artificial condition that may not take place under normal circumstances *in vivo*. In contrast, ocular dominance plasticity and the examples of spatial learning described above do take place *in vivo* and can utilize only normal mechanisms. In addition, plasticity of the vestibulo-ocular reflex, believed to reflect cerebellar LTD, has been prevented by subdural application of the NO scavenger haemoglobin to the cerebellum in rabbit and monkey (Nagao & Ito, 1991). Systemic administration of NOS inhibitors also disrupts the development of on–off sublaminae of the lateral geniculate nucleus in the ferret (Cramer & Sur, 1995). Thus there is ample evidence that NOS inhibition can have drastic effects in complex, *in vivo* systems that do depend on NO production.

A final issue that these experiments did not address is the possibility that inhibiting NO synthesis had morphological effects on the thalamocortical terminals that were not revealed by electrophysiological recordings. For example, a failure of the arbors of axons serving the open eye to expand and of those serving the deprived eye to shrink in the presence of NOS inhibitors might have gone undetected by electrophysiology if appropriate modifications of synaptic strength had rendered deprived eye inputs ineffective and

strengthened inputs from the open eye. Since the known effects of NO inhibition on neural plasticity demonstrated so far are predominantly physiological, involving the ability of synapses to potentiate or depress transmission, it is unlikely that it would have silent morphological effects in the absence of the physiological ones.

What is the function of the NOS present in neocortical neurons? High levels of NOS are expressed in about 1–2% of cortical neurons distributed diffusely across the cortex. These cells are identical to the NADPH–diaphorase-positive neurons that are highly resistant to loss in certain neurodegenerative diseases and ischaemia (Bredt *et al.* 1991). Both in culture and in experimental *in vivo* models of ischaemia, NO, presumably released by these NADPH–diaphorase-positive neurons, has been shown to mediate glutamate neurotoxicity (Koh & Choi, 1988; Dawson, Dawson, London, Bredt & Snyder, 1991). There is also both anatomical and physiological evidence for a contribution of NO to the metabolic regulation of cortical blood flow (for review see Iadecola, 1993). Thus changes in NOS expression in visual cortex seen in response to alterations in the pattern of cortical activity (Aoki *et al.* 1993) appear more likely to reflect a contribution of NO to general cortical maintenance than to plasticity.

Whether NO plays any role in neocortical plasticity will require further study. Nonetheless, the fact that the physiological shift in ocular dominance is both as great and as rapid in the absence of NOS activity as in its presence suggests that the role of NO in ocular dominance plasticity is at most a minor one. The basic finding presented above has been replicated recently by a different group using a similar approach to ours (see preceding paper: Reid, Daw, Czepita, Flavin & Sessa, 1996). Our results show that there exists at least one pathway for developmental cortical plasticity that can occur in the absence of cortical NOS activity.

Antonini, A. & Stryker, M. P. (1993*a*). Development of individual geniculocortical arbors in cat striate cortex and effects of binocular impulse blockade. *Journal of Neuroscience* **13**, 3549–3573.

Antonini, A. & Stryker, M. P. (1993*b*). Rapid remodeling of axonal arbors in the visual cortex. *Science* **260**, 1819–1821.

Aoki, C., Fenstemaker, S., Lubin, M. & Go, C.-G. (1993). Nitric oxide synthase in the visual cortex of monocular monkeys as revealed by light and electron microscopic immunocytochemistry. *Brain Research* **620**, 97–113.

Bannerman, D. M., Chapman, P. F., Kelly, P. A. T., Butcher, S. P. & Morris, R. G. M. (1994). Inhibition of nitric oxide synthase does not impair spatial learning. *Journal of Neuroscience* **14**, 7404–7414.

Bear, M. F., Kleinschmidt, A., Gu, Q. A. & Singer, W. (1990). Disruption of experience-dependent synaptic modifications in striate cortex by infusion of an NMDA receptor antagonist. *Journal of Neuroscience* **10**, 909–925.

Bohme, G. A., Bon, C., Lemaire, M., Reibaud, M., Piot, O., Stutzmann, J.-M., Doble, A. & Blanchard, J.-C. (1993). Altered synaptic plasticity and memory formation in nitric oxide synthase inhibitor-treated rats. *Proceedings of the National Academy of Sciences of the USA* **90**, 9191–9194.

Bredt, D. S., Glatt, C. E., Hwang, P. M., Fotuhi, M., Dawson, T. M. & Snyder, S. H. (1991). Nitric oxide synthase protein and mRNA are discretely localized in neuronal populations of the mammalian CNS together with NADPH diaphorase. *Neuron* **7**, 615–624.

Bredt, D. S. & Snyder, S. H. (1989). Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. *Proceedings of the National Academy of Sciences of the USA* **86**, 9030–9033.

Chapman, P. F., Atkins, C. M., Allen, M. T., Haley, J. E. & Steinmetz, J. E. (1992). Inhibiton of nitric oxide synthesis impairs two different forms of learning. *NeuroReport* **3**, 567–570.

Chetkovich, D. M., Klann, E. & Sweatt, J. D. (1993). Nitric oxide synthase-independent long-term potentiation in area CA1 of hippocampus. *NeuroReport* **4**, 919–922.

Cramer, K. S. & Sur, M. (1995). Activity-dependent remodeling of connections in the mammalian visual system. *Current Opinion in Neurobiology* **5**, 106–111.

Cummings, J. A., Nicola, S. M. & Malenka, R. C. (1994). Induction in the rat hippocampus of long-term potentiation (LTP) and long-term depression (LTD) in the presence of a nitric oxide synthase inhibitor. *Neuroscience Letters* **176**, 110–114.

Dawson, V. L., Dawson, T. M., London, E. D., Bredt, D. S. & Snyder, S. H. (1991). Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. *Proceedings of the National Academy of Sciences of the USA* **88**, 6368–6371.

Dawson, T. M. & Snyder, S. H. (1994). Gases as biological messengers: nitric oxide and carbon monoxide in the brain. *Journal of Neuroscience* **14**, 5147–5159.

Dwyer, M. A., Bredt, D. S. & Snyder, S. H. (1991). Nitric oxide synthase: irreversible inhibition by L-N^G-nitroarginine in brain *in vitro* and *in vivo*. *Biochemical and Biophysical Research Communications* **176**, 1136–1141.

Garthwaite, J. & Boulton, C. L. (1995). Nitric oxide signaling in the central nervous system. *Annual Review of Physiology* **57**, 683–706.

Gribkoff, V. K. & Lum-Ragan, J. T. (1992). Evidence for nitric oxide synthase inhibitor-sensitive and insensitive hippocampal synaptic potentiaion. *Journal of Neurophysiology* **68**, 639–642.

Haley, J. E., Malen, P. L. & Chapman, P. F. (1993). Nitric oxide synthase inhibitors block long-term potentiation induced by weak but not strong tetanic stimulation at physiological brain temperatures in rat hippocampal slices. *Neuroscience Letters* **160**, 85–88.

Hata, Y. & Stryker, M. P. (1994). Control of thalamocortical afferent rearrangement by postsynaptic activity in developing visual cortex. *Science* **265**, 1732–1735.

Hensch, T. K., Crair, M. C., Ruthazer, E. S., Fagiolini, M., Gillespie, D. C. & Stryker, M. P. (1995). Robust two-day ocular dominance plasticity revealed by single-unit recording and intrinsic signal imaging of kitten area 17. *Society for Neuroscience Abstracts* **21**, 2023.

Iadecola, C. (1993). Regulation of the cerebral microcirculation during neural activity: is nitric oxide the missing link? *Trends in Neurosciences* **16**, 206–214.

IRIKURA, K., HUANG, P. L., MA, J., LEE, W. S., DALKARA, T., FISHMAN, M. C., DAWSON, T. M., SNYDER, S. H. & MOSKOWITZ, M. A. (1995). Cerebrovascular alterations in mice lacking neuronal nitric oxide synthase gene expression. *Proceedings of the National Academy of Sciences of the USA* **92**, 6823–6827.

KARA, P. & FRIEDLANDER, M. J. (1995). The role of nitric oxide in modulating the visual response of neurons in cat striate cortex. *Society for Neuroscience Abstracts* **21**, 1753.

KIRKWOOD, A. & BEAR, M. F. (1994). Hebbian synapses in visual cortex. *Journal of Neuroscience* **14**, 1634–1645.

KOH, J. Y. & CHOI, D. W. (1988). Vulnerability of cultured cortical neurons to damage by excitotoxins: differential susceptibility of neurons containing NADPH–diaphorase. *Journal of Neuroscience* **8**, 2153–2163.

MILLER, K. D., CHAPMAN, B. & STRYKER, M. P. (1989). Visual responses in adult cat visual cortex depend on N-methyl-D-aspartate receptors. *Proceedings of the National Academy of Sciences of the USA* **86**, 5183–5187.

MILLER, K. D., KELLER, J. B. & STRYKER, M. P. (1989). Ocular dominance column development: analysis and simulation. *Science* **245**, 605–615.

MONCADA, S., PALMER, R. M. J. & HIGGS, E. A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacological Reviews* **43**, 109–142.

MONTAGUE, P. R., GANCAYCO, C. D., WINN, M. J., MARCHASE, R. B. & FRIEDLANDER, M. J. (1994). Role of NO production in NMDA receptor-mediated neurotransmitter release in cerebral cortex. *Science* **263**, 973–977.

NAGAO, S. & ITO, M. (1991). Subdural application of hemoglobin to the cerebellum blocks vestibuloocular reflex adaptation. *NeuroReport* **2**, 193–196.

O'DELL, T. J., HAWKINS, R. D., KANDEL, E. R. & ARANCIO, O. (1991). Tests of the roles of two diffusible substances in long-term potentiation: evidence for nitric oxide as a possible early retrograde messenger. *Proceedings of the National Academy of Sciences of the USA* **88**, 11285–11289.

OLSON, C. R. & FREEMAN, R. D. (1975). Progressive changes in kitten striate cortex during monocular vision. *Journal of Neurophysiology* **38**, 26–32.

REID, S. N. M., DAW, N. W., CZEPITA, D., FLAVIN, H. J. & SESSA, W. C. (1996). Inhibition of nitric oxide synthase does not alter ocular dominance shifts in kitten visual cortex. *Journal of Physiology* **494**, 511–517.

REITER, H. O., WAITZMAN, D. M. & STRYKER, M. P. (1986). Cortical activity blockade prevents ocular dominance plasticity in the kitten visual cortex. *Experimental Brain Research* **65**, 182–188.

SCHUMAN, E. M. & MADISON, D. V. (1994). Nitric oxide and synaptic function. *Annual Review of Neuroscience* **17**, 153–183.

SHATZ, C. J. & STRYKER, M. P. (1978). Ocular dominance in layer IV of the cat's visual cortex and the effects of monocular deprivation. *Journal of Physiology* **281**, 267–283.

SHIBUKI, K. & OKADA, D. (1991). Endogenous nitric oxide release required for long-term synaptic depression in the cerebellum. *Nature* **349**, 326–328.

STRYKER, M. P. & HARRIS, W. A. (1986). Binocular impulse blockade prevents the formation of ocular dominance columns in cat visual cortex. *Journal of Neuroscience* **6**, 2117–2133.

WIESEL, T. N. (1982). Postnatal development of the visual cortex and the influence of environment. *Nature* **299**, 583–591.

WILLIAMS, J. H., LI, Y. G., NAYA, K. A., ERRINGTON, M. L., MURPHY, K. P. S. J. & BLISS, T. V. P. (1993). The suppression of long-term potentiation in rat hippocampus by inhibitors of nitric oxide synthase is temperature- and age-dependent. *Neuron* **11**, 877–884.

ZHUO, M., SMALL, S. A., KANDEL, E. R. & HAWKINS, R. D. (1993). Nitric oxide and carbon monoxide produce activity-dependent long-term synaptic enhancement in hippocampus. *Science* **260**, 1946–1950.

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